

5 chromatography by anion exchange (Tris-HCl, pH 8.0) followed by cation exchange
chromatography (Acetate buffer at pH 4.5) using NaCl gradients. The elution profile was
monitored by (1) Western blot analysis and (2) by activity assay using the peptide substrate
described in Example 12. For the Hu-Asp2 Δ TM(His)₆, the conditioned medium was
10 dialyzed against Tris buffer (pH 8.0) and purified by sequential chromatography on IMAC
resin followed by anion exchange chromatography.

15 Sequence analysis of the purified Hu-Asp2 Δ TM(His)₆ protein revealed that the signal
peptide had been cleaved [TQHGIPLR].

20 **Example 11. Expression of Hu-Asp2 in CHO cells**

Heterologous expression of Hu-Asp-2L in CHO-K1 cells—The entire coding sequence of
Hu-Asp2 was cloned into the mammalian expression vector pcDNA3.1(+)-Hygro
15 (Invitrogen, Carlsbad, CA) which contains the CMV immediate early promoter and bGH
polyadenylation signal to drive over expression. The expression plasmid,
pcDNA3.1(+)-Hygro/Hu-Asp2, was prepared by alkaline lysis and banding in CsCl and
completely sequenced on both strands to verify the integrity of the coding sequence.

20 Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the ATCC. The
cells were maintained in monolayer cultures in α -MEM containing 10% FCS at 37°C in 5%
CO₂. Two 100 mm dishes of CHO-K1 cells (60% confluent) were transfected with
pcDNA3.1(+)-Hygro alone (mock) or pcDNA3.1(+)-Hygro/Hu-Asp2 using the cationic
liposome DOTAP as recommended by the supplier. The cells were treated with the plasmid
40 DNA/liposome mixtures for 15 hr and then the medium replaced with growth medium
containing 500 Units/ml hygromycin B. In the case of pcDNA3.1(+)-Hygro/Hu-Asp2
transfected CHO-K1 cells, individual hygromycin B-resistant cells were cloned by limiting
dilution. Following clonal expansion of the individual cell lines, expression of Hu-Asp2
protein was accessed by Western blot analysis using a polyclonal rabbit antiserum raised
50

5 against recombinant Hu-Asp2 prepared by expression in *E. coli*. Near confluent dishes of
each cell line were harvested by scraping into PBS and the cells recovered by
centrifugation. The cell pellets were resuspended in cold lysis buffer (25 mM Tris-HCl
10 (8.0)5 mM EDTA) containing protease inhibitors and the cells lysed by sonication. The
soluble and membrane fractions were separated by centrifugation (105,000 x g, 60 min) and
15 normalized amounts of protein from each fraction were then separated by SDS-PAGE.
Following electrotransfer of the separated polypeptides to PVDF membranes, Hu-Asp-2L
protein was detected using rabbit anti-Hu-Asp2 antiserum (1/1000 dilution) and the
20 antibody-antigen complexes were visualized using alkaline phosphatase conjugated goat
anti-rabbit antibodies (1/2500). A specific immunoreactive protein with an apparent Mr
value of 65 kDa was detected in pcDNA3.1(+)/Hygro/Hu-Asp2 transfected cells and not
25 mock-transfected cells. Also, the Hu-Asp2 polypeptide was only detected in the membrane
fraction, consistent with the presence of a signal peptide and single transmembrane domain
in the predicted sequence. Based on this analysis, clone #5 had the highest expression level
30 of Hu-Asp2 protein and this production cell lines was scaled up to provide material for
purification.

35 *Purification of recombinant Hu-Asp-2L from CHO-K1/Hu-Asp2 clone #5*—In a
typical purification, clone #5 cell pellets derived from 20 150 mm dishes of confluent cells,
were used as the starting material. The cell pellets were resuspended in 50 ml cold lysis
40 buffer as described above. The cells were lysed by polytron homogenization (2 x 20 sec)
and the lysate centrifuged at 338,000 x g for 20 minutes. The membrane pellet was then
45 resuspended in 20 ml of cold lysis buffer containing 50 mM β -octylglucoside followed by
rocking at 4°C for 1 hr. The detergent extract was clarified by centrifugation at 338,000 x g
for 20 minutes and the supernatant taken for further analysis.

5 The β -octylglucoside extract was applied to a Mono Q anion exchange column that
was previously equilibrated with 25 mM Tris-HCl (pH 8.0)/50 mM β -octylglucoside.
Following sample application, the column was eluted with a linear gradient of increasing
10 NaCl concentration (0-1.0 M over 30 minutes) and individual fractions assayed by Western
5 blot analysis and for β -secretase activity (see below). Fractions containing both Hu_Asp-
2L immunoreactivity and β -secretase activity were pooled and dialyzed against 25 mM
15 NaOAc (pH 4.5)/50 mM β -octylglucoside. Following dialysis, precipitated material was
removed by centrifugation and the soluble material chromatographed on a MonoS cation
20 exchange column that was previously equilibrated in 25 mM NaOAc (pH 4.5)/ 50 mM β -
10 octylglucoside. The column was eluted using a linear gradient of increasing NaCl
concentration (0-1.0 M over 30 minutes) and individual fractions assayed by Western blot
25 analysis and for β -secretase activity. Fractions containing both Hu-Asp2 immunoreactivity
and β -secretase activity were combined and determined to be >90% pure by SDS-
30 PAGE/Coomassie Blue staining.

15 **Example 12. Assay of Hu-Asp2 β -secretase activity using peptide substrates**

β -secretase assay— β -secretase activity was measured by quantifying the hydrolysis of a
35 synthetic peptide containing the APP Swedish mutation by RP-HPLC with UV detection.
Each reaction contained 50 mM Na-MES (pH 5.5), 1% β -octylglucoside, peptide substrate
40 (SEVNLDAEFR, 70 μ M) and enzyme (1-5 μ g protein). Reactions were incubated at 37 °C
20 for various times and the reaction products were resolved by RP-HPLC using a linear
gradient from 0-70 B over 30 minutes (A=0.1% TFA in water,
45 B=).1%TFA/10%water/90%AcCN). The elution profile was monitored by absorbance at
214 nm. In preliminary experiments, the two product peaks which eluted before the intact
50 peptide substrate, were confirmed to have the sequence DAEFR and SEVNL using both